Epidemiology of Tuberculosis with comparison of the efficacy of culture, real-time PCR and direct microscopy in the diagnosis of tuberculosis

Epidemiology of tuberculosis

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Abstract

Aim: Despite the developments every passing year, tuberculosis is still a major public health problem in Turkey and in the world. Early and correct diagnosis is important for the control of the disease. The aim of this study is to investigate the diagnostic efficiency of the culture (Löwenstein Jensen), real-time PCR (Abbott m2000rt, Bosphore MTBC Detection Kit) and direct microscopic examination of acid-resistant bacillus stained with Ehrlich-Ziehl-Neelsen (EZN), and the study was performed by evaluating the samples coming to our laboratory retrospectively over a 4-year period.

Material and Methods: The samples sent to our mycobacteriology laboratory between January 2016 and December 2019 were evaluated retrospectively. The effects of direct microscopy and real-time PCR method were compared based on culture as a reference method.

Results: Sensitivity, specificity, positive predictive value and negative predictive value of direct microscopy were calculated as 45%, 99.9%, 96.4%, 96.9%, respectively, in pulmonary samples and as 21.7%, 100%, 98.8%, 98.8%, respectively, in extrapulmonary samples. Sensitivity, specificity, positive predictive value and negative predictive value of real-time PCR were calculated as 80%, 98.3%, 98.8%, 97.3%, respectively, in pulmonary samples and as 62.5%, 99.2%, 50%, 99.5%, respectively, in extrapulmonary samples.

Discussion: Although direct microscopic examination method is a cheap, simple, fast and highly specific test, its sensitivity is low. Therefore, patients with suspected tuberculosis should be evaluated together with culture, besides direct microscopy. It was observed that the sensitivity of direct microscopy was lower in extrapulmonary samples compared to pulmonary samples. The real-time PCR method has high sensitivity and specificity, and also gives fast results.

Keywords

Ehrlich-Ziehl-Neelsen; Epidemiology; Löwenstein Jensen; Real-Time PCR; Tuberculosis

Introduction

It is estimated that 1.7 billion people in the world are infected with tuberculosis, and it is expected that tuberculosis disease developed in a certain period of their lives in 5-10% of the infected population [1]. Approximately 10 million people worldwide suffer from tuberculosis each year, and 1.45 million people died from tuberculosis worldwide in 2018 (available at: www.who.int). Most of the deaths occurred in patients who are coinfected with HIV and have multiple drug resistance [2]. Although the incidence of tuberculosis and deaths from tuberculosis have been declining each year, health problems that occur due to tuberculosis still remain serious in endemic regions (available at: www.who.int). According to the statistics of the tuberculosis department, the incidence of tuberculosis decreases each year in our country, and the incidence in 2018 is 14.1 (in 100.000), the number of cases is11,786 people (available at: www.toraks.org.tr).

Although tuberculosis can occur with many different clinical pictures, it is basically divided into pulmonary and extrapulmonary tuberculosis according to the involvement region of the disease, and the vast majority of cases is pulmonary tuberculosis [3]. It is important to detect the disease early and correctly in order to isolate patients and not to delay the treatment. Early diagnosis and appropriate treatment approach reduce tuberculosis transmission [1]. Bacteriological confirmation, including the detection of acid-resistant bacilli on microscopic examination and the growth of tuberculosis bacillus in culture, is important in people with suspected tuberculosis due to the low specificity of clinical and radiological findings (available at www.hsgm.saglik.gov.tr).

Although many methods can be applied in the diagnosis of tuberculosis, tuberculosis culture is the gold standard method [4]. It is sufficient that the sample contains 10-100/ml bacillus to produce tuberculosis bacillus in culture [5]. Although direct microscopy is simple, cheap, fast and highly specific, it is a diagnostic tool with low sensitivity [6]. The type and quality of the sample, the experience of the assessor, the bacillus load in the sample, the differences inthe procedures applied to the sample directly affect the sensitivity of the microscopy [5]. In order for the bacillus to be visible in the microscopic examination, the sample must contain 5,000-10,000 / ml tuberculosis bacillus [7]. There are different staining methods during sample preparation for direct microscopic examination, but EZN staining is the most commonly used method [8]. Fluorescent staining methods have higher sensitivity rates compared to the EZN method and are preferred in centers that especially have high numbers of samples due to the shortening of the evaluation time [2,8]. Due to the low sensitivity of microscopic examination and the late outcome of culture methods, many molecular methods have been developed for use in the diagnosis of tuberculosis [9]. These molecular methods are mainly based on nucleic acid amplification and include various procedures such as polymerase chain reaction (PCR), strand displacement amplification (SDA), transcription-mediated amplification (TMA), Ligase chain reaction (LCR) [6]. PCR technology has started to be used in the diagnosis of tuberculosis firstly with conventional PCR methods, and then with real-time PCR, which was developed later [10, 11]. The real-time PCR method is faster than conventional PCR methods and has a higher repeatability [12]. In the diagnosis of tuberculosis, although real-time PCR has advantages such as fast result time, high sensitivity and specificity, it also includes disadvantages such as the needi for trained personnel and high cost [6]. In this study, it was aimed to compare the diagnostic efficiency of diagnostic methods by evaluating direct examination, tuberculosis culture and real-time PCR results of the patients whose samples were sent to our laboratory with the pre-diagnosis of tuberculosis between 2016-2019.

Material and Methods

Arb direct examination, tuberculosis culture and real-time PCR results of the patients whose samples were sent to our mycobacteriology laboratory with the pre-diagnosis of tuberculosis between 2016-2019 at the Recep Tayyip Erdoğan University Faculty of Medicine Education and Research Hospital were examined retrospectively. The number of pulmonary samples was 2282 (%58.8) (sputum, bronchoalveolar lavage, tracheal aspirate), and 1595 (41.1%) samples were extrapulmonary (abscess, tissue, urine, empty, pleural fluid, paracentesis, joint fluid and others).

In the first stage, homogenization and decontamination were applied to the samples from contaminated areas such as sputum, bronchoalveolar lavage, tracheal aspirate, wound, tissue, abscess. Urine samples were centrifuged at 3000 rpm for 20 minutes and then subjected to homogenization and decontamination processes. N-Acetyl L-Cysteine was used in the homogenization process and NaOH was used in the decontamination process. Through homogenization and decontamination processes, it is aimed to make the dense samples homogeneous and to make the tuberculosis bacillus visible by removing the microorganisms from the flora. Samples to which homogenization and decontamination processes were applied, were neutralized with phosphate buffer. Cerebrospinal fluid, joint fluid, pleural fluid and paracentesis fluid samples were not subjected to decontamination processes. All samples were finally centrifuged and concentrated and made ready for processing.

Arb direct examination, tuberculosis culture, and real-time PCR were applied to samples prepared among consideration of the clinical request.

Direct Microscopic Examination:

The presence of acid-resistant bacilli was investigated using preparations, which were stained with the Erlich Ziehl-Neelsen (EZN) staining method for direct microscopic examination. Acid- resistant bacilli samples were scored according to the standards of the American Thoracic Society and reported as "Acid resistant bacillus [3].

Tuberculosis Culture:

Löwenstein Jensen medium, a solid, egg-based medium, was used for tuberculosis culture. Samples planted on Löwenstein Jensen medium were incubated for up to 8 weeks and were checked for reproductive follow-up, 2 times in the first week, and then at least once a week until the 8th weeks. The presence of acid-resistant bacillus was scanned on positive growth obtained medium stained with the Erlich Ziehl-Neelsen (EZN) staining method and samples, showing acid-resistant bacillus

were resulted as "reproduction has occurred in Löwenstein Jensen medium." The medium, on which no reproduction was obtained at the end of the 8-week incubation period was lastly stained with the Erlich Ziehl-Neelsen (EZN) staining method, and after that, if no acid-resistant bacillus was detected on samples, result was as follows: reproduction did not occur in the Löwenstein Jensen medium (available at: www.hsgm.saglik.gov.tr).

Real-Time PCR:

Samples sent between the years 2016-2017 were evaluated with Abbott m2000rt (Abbott Molecular, USA), while samples sent between the years 2018-2019 were with Bosphore MTBC Detection Kit (Geneworks Anatolia, Turkey). The working procedure has been carried out according to the manufacturer's instructions.

Abbott m2000rt (Abbott Molecular, USA): The procedure was started with a sample inactivation step to reduce the risk of infection regarding clinical samples. The mixture formed using 1.5 ml of inactivation reagent (0.4 M NaOH, 60% Isopropanol, 0.18% Tween-20) according to the 0.5 ml of the sample, was incubated for 1 hour at room temperature. Thereafter, automated DNA isolation was performed with the Abbott m2000sp device (Abbott Molecular, USA) using the mSample Preparation DNA kit (Abbott Molecular, USA). 25 µL of amplification master mix were mixtured with the taken 25 µL of the eluted sample. The PCR reaction was performed by transferring the prepared mixture to the Abbott m2000rt (Abbott Molecular, USA) device. One positive and one negative control were used for each sample.

Bosphore MTBC Detection Kit (Anatolia Geneworks, Türkiye): After the samples, for which the real-time PCR test was requested were ready for process, then 750 ml was received from samples for DNA extraction in the first stage, and DNA isolation process was completed using the 'Magnesia 16 Nucleic Acid Isolation Kit' (Anatolia Geneworks, Turkey). 18µL of DNA from the DNA isolation samples was taken into the tubes, adding 22.8 µL of the PCR master mix and 0.2 µL of the internal control. During the PCR reaction, the first denaturation of DNA occurred at 95 °C for 14.5 minutes. This was followed by a denaturation at 97 ° C for 0.5 minutes and a total of 50 cycles, including binding and synthesis at 53 °C for 2 minutes in. After the 50th cycle, an incubation period was performed at 22 °C for 5 minutes. After the thermal cycle, amplification curves were evaluated. Patients' results with similar logarithmic curves with the clinical sample in the internal control were evaluated as positive, and those without logarithmic curve as negative.

Ethics Considerations

Ethics committee approval for this study was obtained from the Recep Tayyip Erdoğan University Non-Interventional Ethics Committee (Approval no:2020/208). The study was conducted in line with the ethical principles of the Declaration of Helsinki

Results

Demographic Data:

A total of 3877 patients were included in the study, 2653 (68.4%) of them were male and 1224 (31.6%) were female. When the distribution of samples was analyzed, it was observed that the largest group was the Chest Diseases service, with

2325 (60%) patients; 889 of these patients belonged to the year 2016, 834 to 2017, 991 to 2018 and 1163 belonged to 2019. The average age of the patients was determined as 64. The average age of male patients was 63.5, and the average age of female patients was 65.2.

Direct Microscopic Inspection Findings:

Direct microscopy was requested from 3834 of the patients; 2281 of the samples were grouped as pulmonary, and 1553 as extrapulmonary. Direct microscopic examination findings are summarized in Table 1.

In pulmonary samples, the sensitivity, specificity, positive predictive value and negative predictive values were calculated as 45%, 99.9%, 96.4% and 96.9%, respectively. In extrapulmonary samples, the sensitivity, specificity, positive predictive value and negative predictive values were calculated as 21.7%, 100%, 98.8%, 98.8%, respectively (Table 3).

Culture Methods Findings:

Tuberculosis culture results were positive in 146 (3.8%) samples and negatively in the 3731 (96.2%) samples

Real-Time PCR Findings:

Real-time PCR was requested from 1387 patients. These samples were grouped as 709 pulmonary and 678 as extrapulmonary. Real-time PCR findings are summarized in Table 2.

Table 1. Direct Microscopy Results

Direct Microscopy		Positive	Negative
Pulmonary (2281)	Positive(122)	55	67
	Negative(2159)	2	2157
Culture			
Extrapulmonary (1553)	Positive(23)	5	18
	Negative(1530)	0	1530

Table 2. Real-time PCR results

Real- time PCR		Positive	Negative
Pulmonary (709)	Positive(40)	32	8
	Negative(669)	11	658
Culture			
Extrapulmonary (678)	Positive(8)	5	3
	Negative(670)	5	665

Table 3. Comparison of sensitivity, specificity, positive predictive value and negative predictive values of Real-Time PCR with direct microscopy

		Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)
Pulmonary	Direct Microscopy	45	99.9	96.4	96.9
	Real-Time PCR	80	98.3	98.8	97.3
Extrapulmonary	Direct Microscopy	21.7	100	98.8	98.8
	Real-Time PCR	62.5	99.2	50	99.5

In pulmonary samples, the sensitivity, specificity, positive predictive value and negative predictive values were calculated as 80%, 98.3%, 98.8%, 97.3%, respectively. In extrapulmonary samples, the sensitivity, specificity, positive predictive value and negative predictive values were calculated as 62.5%, 99.2%, 50%, 99.5%, respectively (Table 3).

Discussion

Tuberculosis disease remains valid as a major public health problem in Turkey and in the world, despite all the developments. Early and accurate diagnosis is important for disease control. Different methods are being developed to define the tuberculosis bacillus every passing year. The sensitivity and specificity of these diagnostic methods vary among each other. Direct microscopy is one of the most commonly used methods to evaluate the presence of arb. The main disadvantage of direct microscopic examination is its low sensitivity rate. The sensitivity and specificity of direct microscopic examination were calculated as 82.3% and 99.7%, retrospectively in the study performed with pulmonary samples in Brazil [9]. In another study that performed in 2016 in Turkey, 62 456 samples were examined and the sensitivity, specificity, positive predictive value, and negative predictive values of the direct microscopy method reported as 32.8%, 99.4%, 87.5%, 91.4 %, respectively [8]. The effectiveness of direct microscopic examination varies among pulmonary and extrapulmonary samples. In the study conducted in our country by Karadag et al., the sensitivity, specificity, positive predictive value and negative predictive values were calculated as 71.4%, 98.8%, 83.3%, 97.6%, respectively, in pulmonary samples and as 24%, 98.3%, 42.8%, 96.2%, respectively, in extrapulmonary samples [4]. The sensitivity, specificity, positive predictive value and negative predictive values of the direct microscopic examination method were calculated as 60%, 100%, 100%, 96.5%, respectively in pulmonary samples and as 32.3%, 99.5%, 76.9%, 96.9%, respectively in extrapulmonary samples [5]. The gold standard method in the diagnosis of tuberculosis is tuberculosis culture. The main disadvantage of the method is that it gives results in 6-8 weeks. In recent years, liquid-based culture methods have been developed that yield earlier results. Liquid-based culture systems produce results in a shorter time than solid media, but they are more costly [21]. Both the sensitivity and specificity of the culture are higher than microscopic examinations [22].

Molecular tests have been started to be used in the diagnosis of tuberculosis over time. Molecular methods contain systems including different methods. Recently, nucleic acid amplification tests have become widely used. Although many nucleic acid amplification tests are available, the most widely used test is real-time PCR. Real-time PCR has a high sensitivity and specificity compared to direct microscopic examination [6]. The sensitivity, specificity, positive predictive and negative predictive values of real-time PCR were calculated as 82.3%, 97.6%, 93.3%, 93%, respectively, in the study performed by Bajrami et al [15].

The effectiveness of real-time PCR varies between pulmonary and extrapulmonary samples. There are studies that report the sensitivity of the real-time PCR as 59%, 66.9% and 68% when performing research on pulmonary samples [12, 16, 17].

PCR sensitivities were calculated as 61.1% and 65.6% in a study comparing two different PCR systems in extrapulmonary samples [18]. In meta-analyzes, it was published that the sensitivity of real-time PCR method was 82%, 80.8% in pulmonary samples and was 70%, 58.6% in extrapulmonary samples [10, 11].

Conventional PCR methods were compared with real-time PCR methods in the study performed by Tortoli et al. In this study sensitivity of conventional PCR was reported as 81% in pulmonary samples, and as 60.3% in extrapulmonary samples and also the sensitivity of real-time PCR reported as 81.1% in pulmonary samples and as 64.2% in extrapulmonary samples [19].

Our study had limitations such as not performing direct microscopic examination, culture and real-time PCR for all samples and not performing advanced identification for all cultures, which showed growth. When advanced identification is not performed, mycobacterium growths other than tuberculosis are considered false positive, and the patient can be treated incorrectly.

Conclusions:

Although the direct microscopic examination method is cheap, simple, rapid and highly specific test, its sensitivity is low. Direct microscopic examination has a wide sensitivity range in the studies we examined. For this reason, patients with suspected tuberculosis should be evaluated with culture besides direct microscopy. It was observed the sensitivity of the direct microscopic examination was lower in extrapulmonary samples compared to pulmonary samples. Besides providing rapid results, the real-time PCR method has high sensitivity and specificity as well.

In the studies we examined, it was shown that the sensitivity of PCR is high compared to direct microscopic examination. It was observed that the sensitivity of PCR was lower in extrapulmonary samples compared to pulmonary samples. Culture methods remain valid as reference methods, but advanced identification should be performed on samples, which have grown positively Some patients were treated with antibiotics unnecessarily because they were misdiagnosed due to late results of the culture methods, and some tuberculosis patients could cause disease spread because of not being diagnosed during this period.

In the light of all this information, real-time PCR is evaluated as a suitable method for use due to its high sensitivity among early diagnosis methods, although it has a high cost.

Conflict of interest

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